MEMBRANE-DEGRADING ENZYMES IN THE LEAVES OF *SOLANUM TUBEROSUM*

Abstract—When homogenates of potato leaves were prepared under conditions which are typical for organelle isolation (pH 7.5 and 4°), membrane lipids underwent rapid hydrolysis (17% of phosphatidylcholine was hydrolysed in 2 hr). Leaves of 41 potato cultivars were surveyed for phospholipase activity to determine whether certain cultivars might be more suitable for the preparation of organelles. Phospholipase activities ranged from 1.04 to 11.60 μmol/min · g fr. wt and *p*-nitrophenyl palmitate hydrolase activity ranged from 0.0119 to 0.0502 μmol/min · g fr. wt. These phospholipase values were several hundred-fold higher than previously reported for potato leaves and nearly as high as in potato tubers. Most of the phospholipase activity in leaves was soluble and not membrane-associated as previously reported.

INTRODUCTION

Lipolytic acyl hydrolases have been extensively studied in potato tubers and bean leaves [1]. In 1979 Matsuda and Hirayama [2] reported galactolipase and phospholipase levels in the leaves of several Japanese potato cultivars. They also reported that potato leaves contained much less (400-fold) phospholipase activity than potato tubers [2]. An acyl hydrolase was subsequently purified from potato leaves and found to hydrolyse both galactolipids and phospholipids [3]. Another laboratory recently reported that 26–37% of the phospholipids in potato leaf homogenates were hydrolysed after 2 hr at 0–1° [4].

This study was undertaken in order to determine whether potato leaves contain enough membrane-degrading enzymes to cause problems during the isolation of organelles and membrane vesicles. A survey of phospholipase activity in the leaves of common potato cultivars was also conducted in order to investigate whether certain cultivars contained significantly less activity, thus making them more suitable for subcellular fractionation studies.

RESULTS

In order to determine whether the breakdown of membrane lipids would be a serious problem during the preparation of organelles from potato leaves, homogenates were prepared from leaves (cv. Kennebec) and analysed (Table 1). Under conditions normally used for organelle isolation (pH 7.5 and 4°), 17% of the phosphatidylcholine was hydrolysed after 2 hr. Membrane breakdown was more rapid when homogenates were buffered at pH 6.0 or when incubated at 20°. These results indicated that extensive breakdown of membrane lipids did occur with Kennebec leaves under conditions normally used for organelle isolation. It is likely that homogenization with a lower concentration of buffer (< 0.1 M), as is common for organelle isolation, would result in a lower pH after homogenization and even more extensive membrane breakdown. The remainder of the study was designed to investigate whether the leaves of certain cultivars contained significantly less phospholipase activity than those of Kennebec, thus making them more suitable for subcellular fractionation studies.

Preliminary experiments were conducted with Kennebec leaves in order to determine the optimal conditions for enzyme preparation and for assaying phospholipase activity in potato leaves. When leaves were ground in phosphate buffer alone there was very rapid browning (within 30 min) of the homogenates and phospholipase activity was low in the supernatant (Table 2). The addition of various reducing agents prevented browning and resulted in 10-fold more soluble phospholipase activity. These results indicate that phospholipase activity is probably inactivated by polyphenol oxidase or its toxic products. It is also possible that these reductants also retain enzyme activity by preventing the formation of disulphide bonds. Because sodium metabisulphite seemed to prevent browning longer than dithiothreitol or β-mercaptoethanol, it was added to the grinding buffer in all subsequent experiments. Since 83% of the phospholipase activity was found in the 20 000 *g* supernatant, phospholipase activity was surveyed only in the supernatants and not in the pellets. When the 20 000 *g* supernatant was subjected to further centrifugation (100 000 *g* for 60 min) all of the activity remained in the supernatant indicating that the enzyme was soluble. Using Kennebec supernatant, maximal phospholipase activity was obtained with 1 mM phosphatidylcholine, 0.5% Triton X-100† and 100 mM potassium phosphate pH 6.0. Because these

*Agricultural Research Service, U.S. Department of Agriculture.

†Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Table 1. Breakdown of phosphatidylcholine in homogenates of Kennebec potato leaves

Grinding medium buffered with	Temperature (°)	nmol PC/ml at 0 hr	nmol PC/ml after 2 hr	% hydrolysis
0.1 M MES, pH 6.00	4	158	96	39
	20	155	14	90
0.1 M HEPES, pH 7.50	4	161	134	17
	20	154	20	87

After homogenization the pH of the MES homogenate was 6.02 and the pH of the HEPES homogenate was 7.19.

Table 2. Effect of addition of reducing compounds during homogenization of potato leaves

Homogenization medium (0.1 M potassium phosphate buffer pH 7.0)	Centrifugation fraction (20000 *g*)	Browning (visual estimate)	Phospholipase activity μmol/min · g fr. wt
Buffer only	Supernatant	Yes	0.110
	pellet		0.230
Buffer + 2 mM sodium metabisulphite	Supernatant	No	1.232
	pellet		0.255
Buffer + 5 mM dithiothreitol	Supernatant	No	1.298
Buffer + 5 mM β-mercaptoethanol	Supernatant	No	1.165

conditions were very similar to the optimal conditions in the only other comparable study [3], they were used to assay for phospholipase activity in the leaves of all cultivars.

In the survey (Table 3), total phospholipase activity ranged from 1.04 to 11.60 μmol/min · g fr. wt and the specific activity ranged from 0.075 to 0.682 μmol/min · mg protein. The activity of *p*-nitrophenyl palmitate hydrolase (PNP hydrolase) was very low and ranged from 0.0119 to 0.0502 μmol/min · g fr. wt. There was no direct correlation between phospholipase and PNP hydrolase activities, but there was a trend toward an increase in PNP hydrolase activity when the cultivars were listed in order of increasing phospholipase activities. The concentration of soluble protein ranged from 9.03 to 21.69 mg/g fr. wt.

DISCUSSION

The results of this study indicate that potato leaves contain much higher levels of membrane-degrading enzymes than previously recognized [1, 2, 3]. The rates of membrane lipid degradation which were observed in Table 1 with Kennebec leaves are comparable to those reported for Russian varieties [4]. The two previous studies reported a total phospholipase activity of 0.009 μmol/min · g fr. wt [2] and a specific activity of 0.0003 μmol/min · mg protein [3]. The current results reveal that potato leaves contain nearly as much phospholipase activity as potato tubers [1] and much more than bean leaves [1]. Although the levels of phospholipase activity are comparable in potato leaves and tubers, this study demonstrates that leaves contain much less (100-fold) *p*-nitrophenyl palmitate hydrolase activity than tubers [1]. There was a 10-fold range of phospholipase activities in the leaves of the 41 cultivars tested in this report. However, even the leaf homogenates of those cultivars with the lowest levels of phospholipase have the potential ability (at pH 6.0 and 30°) to degrade all of their phospholipids in less than 5 min (based on an average phospholipid content of 3 mg/g fr. wt as measured by Rodionov and Zakharova [4]). Even when working at pH 7.5 and 4° with a variety containing moderate levels of enzyme (Table 1), the membrane degradation caused by such high levels of phospholipase would probably make potato leaves a very difficult tissue from which to isolate organelles and membranes. It may also explain some of the problems that have been encountered in generating and fusing protoplasts from potato leaves [6]. Our laboratory is now in the process of screening potential chemical inhibitors of potato leaf phospholipase activity, as none have previously been reported.

This study also revealed that most (83%) of the phospholipase activity in potato leaves is soluble. This is in contrast to a previous report [3] which stated that the activity was particulate and could only be solubilized with acetone.

There are several possible reasons why other laboratories have reported much lower (several hundred-fold) levels of lipolytic enzymes in potato leaves than were measured in this study. The simplest explanation is that the leaves of Japanese potato cultivars may contain less phospholipase activity than the leaves of North American

Table 3. Levels of lipolytic enzymes in potato leaves*

Cultivar	Phospholipase (μmol/min · g fr. wt)	Phospholipase (μmol/min · mg)	PNP hydrolase (μmol/min · g fr. wt)	Protein (mg/g fr. wt)
Butte	1.04	0.075	0.0160	13.80
Denali	1.17	0.130	0.0139	9.03
Campbell-12	2.28	0.173	0.0190	13.21
Lemki	2.29	0.217	0.0198	10.53
Cherokee	2.35	0.240	0.0176	9.78
Centennial	2.88	0.256	0.0154	11.25
Red LaSada	2.92	0.254	0.0187	11.51
White Rose	2.93	0.286	0.0119	10.23
Superior	3.11	0.321	0.0147	9.70
La Rouge	3.28	0.259	0.0194	12.65
Crystal	3.29	0.229	0.0204	14.37
Katahdin	3.57	0.280	0.0315	12.73
Norgold Russett	3.57	0.282	0.0211	12.65
Saco	3.59	0.263	0.0206	13.67
Cobbler	3.61	0.224	0.0256	16.11
Highlat	3.67	0.320	0.0171	11.48
Bounty	3.83	0.315	0.0204	12.16
Red Pontiac	3.86	0.257	0.0181	15.02
Wausseon	3.92	0.293	0.0230	13.39
Russett Burbank	4.26	0.358	0.0303	11.90
Dakchip	4.27	0.299	0.0286	14.26
Belchip	4.78	0.364	0.0187	13.12
Kennebec	5.07	0.487	0.0216	10.41
GreenMountain	5.08	0.356	0.0214	14.26
Monoma	5.35	0.306	0.0410	17.46
Abnaki	5.50	0.307	0.0193	17.92
Campbell-13	5.55	0.333	0.0191	16.65
Ontario	5.99	0.432	0.0311	13.85
Bel Rus	6.38	0.349	0.0443	18.26
Alaska Red	6.55	0.376	0.0182	17.41
Buckskin	6.69	0.451	0.0255	14.83
Campbell-11	7.05	0.433	0.0421	16.30
La Chipper	7.40	0.410	0.0303	18.06
Sebago	7.55	0.462	0.0333	16.34
Atlantic	7.72	0.508	0.0394	15.20
Pungo	8.01	0.538	0.0292	14.89
Norchip	8.34	0.450	0.0502	18.55
Desiree	9.39	0.578	0.0460	16.23
Bison	9.66	0.445	0.0485	21.69
Hudson	10.24	0.636	0.0276	16.10
Houma	11.60	0.682	0.0307	17.00

*Data represent the averages of two or three separate experiments (separate leaf samples, homogenizations and enzyme assays) for each cultivar. The deviations from the mean were less than 30% for all cultivars.

cultivars. An alternative explanation may involve the inactivation of phospholipase by polyphenol oxidase or its toxic quinone products. The data in Table 2 indicate that when polyphenol oxidase activity is controlled, 10 times more phospholipase activity was detected. In the previous reports [2, 3], leaves were homogenized in 5 mM Tris buffer (pH 7.0) with no reducing agents, so it was likely that some inactivation occurred during enzyme preparation.

EXPERIMENTAL

Materials. Seed potato tubers (*Solanum tuberosum*) of each cultivar were obtained from Aroostoock Farms, USDA-ARS, Presque Isle, Maine in the autumn of 1982. Tubers were stored at 4° for 4 months. They were then allowed to sprout at 25° and were planted in 6 in. clay pots in commercial potting soil. Plants were grown in a greenhouse with supplemental light from 1000 W metal halide lamps. Phosphatidylcholine ([^{14}C]dipalmitoyl) was obtained from New England Nuclear. All other reagents were obtained from Sigma.

Lipid analysis of leaf homogenates. Leaf homogenates were prepared by grinding 5 g leaves (cv. Kennebec) (3–4 cm in length) with 20 ml 0.3 M sucrose, 0.1 M buffer (MES pH 6.0 or HEPES pH 7.5), 2 mM EDTA, 5 mM dithiothreitol and 5 mM β-mercaptoethanol in a chilled mortar and pestle. The homogenate was filtered through two layers of cheesecloth and incubated in a 4° water bath. Triplicate 1 ml samples were removed immedi-

ately (0 hr) and after 2 hr. Lipids were extracted with 7 ml hexane–isopropanol (3:2) and 5 ml 6.7% Na_2SO_4, spotted on silica gel G TLC plates, developed in $CHCl_3$–MeOH–H_2O (65:25:4), and visualized with I_2. The spots which co-chromatographed with phosphatidylcholine standards were scraped and subjected to phosphorus analysis [7].

Enzyme preparation. Leaves were harvested from internodes 5–8 at 4–6 weeks after planting. Samples (2.5 g) were homogenized in a procelain mortar and pestle in 25 ml 0.1 M K-Pi buffer (pH 7.0) and 2 mM sodium metabisulphite at 0°. The homogenate was filtered through two layers of cheesecloth and centrifuged at 20 000 *g* for 30 min. The supernatant was collected and enzyme activities were measured within 4 hr.

Enzyme assays. Phospholipase activity was measured with [^{14}C]phosphatidylcholine (PC). The substrate was prepared by sonicating labelled PC ([1-^{14}C]dipalmitoyl) and unlabelled PC in 1.0% Triton X-100 in a bath sonicator (Bransonic 12, Branson, Shelton, U.S.A.) until the emulsion became crystal clear. The standard reaction mixture (1 ml) contained 1 mM PC-dipalmitoyl (50 000 dpm), 0.5% Triton X-100, 100 mM Tricine–NaOH (pH 8.5), 1 mM $CaCl_2$ and 2–5 μl enzyme. Two different enzyme concentrations were tested for each cultivar. The reaction vials were incubated in a shaking water bath (140 rpm) at 30° for 30 min. The enzymatic reactions were simultaneously stopped and extracted by adding 50 μl HOAc, 7 ml hexane–isopropanol (3:2) and 5 ml 6.7% Na_2SO_4. The lipids were removed in the top phase and dried under a stream of N_2. Oleic acid and PC (soybean) (50 μg of each) were added as carriers and the lipid samples were spotted on 250 μm silica gel G TLC plates and developed in hexane–Et_2O–HOAc (70:30:1.5). The free fatty acid and PC spots were visualized with I_2. The radioactivity in the two regions was quantitated with a Berthold LB 283 linear analyser. The per cent conversion of [^{14}C]PC to ^{14}C labelled free fatty acid was used to calculate enzyme activity based on the initial amount of substrate (1 μmol).

Esterase activity. The activity of *p*-nitrophenyl palmitate hydrolase (PNP hydrolase) was measured spectrophotometrically. The reaction mixture (1 ml) contained 0.40 mM PNP, 0.2% Triton X-100, 50 mM K-Pi (pH 8.0) and 2–20 μl enzyme. The change in *A* at 405 nm was measured continuously for 3–4 min with a Beckman Model 35 spectrophotometer. Three different enzyme concns were tested for each cultivar.

Protein assay. Samples of tuber supernatants were mixed with an equal vol. of 10% trichloroacetic acid and incubated at 0° for 18 hr. The mixture was then centrifuged at 5000 *g* for 10 min. The pellet was resuspended in 0.1 N NaOH and protein was assayed by the Lowry–Markwell procedure [5].

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